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Differences in intrinsic efficacy of benzodiazepines are reflected in their concentration-EEG effect relationship

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1 The relevance of EEG effect parameters as a measure of the central nervous system effects of benzodiazepines was evaluated. The concentration-EEG effect relationships of the benzodiazepine agonist midazolam, partial agonist bretazenil, antagonist flumazenil and inverse agonist Ro 19-4603 were quantified and compared with the intrinsic efficacy and affinity of these compounds at the γ-aminobutyric acid (GABA)-benzodiazepine receptor complex.

2 The pharmacokinetics and pharmacodynamics of the compounds were determined after a single intravenous bolus administration of $5\,mg\,kg^{-1}$ midazolam, $2.5\,mg\,kg^{-1}$ bretazenil, $10\,mg\,kg^{-1}$ flumazenil or $2.5\,mg\,kg^{-1}$ Ro 19-4603 to male Wistar derived rats. In a separate experiment the distribution between blood, cerebrospinal fluid and brain concentrations of these compounds was determined. A sensitive assay was developed to measure bretazenil and Ro 19-4603 concentrations in small samples of biological fluids.

3 The benzodiazepine-induced changes in amplitudes in the 11.5–30 Hz frequency band, as determined by aperiodic analysis, was used as EEG effect measure. Concentration-EEG effect relationships were derived by a pharmacokinetic-pharmacodynamic modelling procedure and in the case of midazolam, bretazenil and Ro 19-4603 successfully quantified by the sigmoidal E_{max} model. Large differences in maximal effect of midazolam ($E_{max} = 73 \pm 2\,\mu Vs^{-1}$), bretazenil ($E_{max} = 19 \pm 1\,\mu Vs^{-1}$) and Ro 19-4603 ($E_{max} = -6.5 \pm 0.4\,\mu Vs^{-1}$) were observed, reflecting their differences in intrinsic efficacy. A close correlation was found between the EC_{50} values based on free drug concentration and receptor affinity as determined by displacement of [^{3}H]-flumazenil in a washed brain homogenate at 37°C. In the concentration range of receptor saturation flumazenil did not produce any changes in the EEG effect measure.

4 The study demonstrated that the change in amplitudes in the 11.5–30 Hz frequency band of the EEG is a relevant measure of the pharmacological effect intensity of benzodiazepines, because it seems to reflect their affinity and intrinsic efficacy at the central GABA-benzodiazepine receptor complex.

Keywords: Pharmacokinetics; pharmacodynamics; modelling; benzodiazepines; EEG; efficacy; partial agonist; antagonist; inverse agonist

Introduction

Knowledge of the relationships that can describe and predict the time course of drug action *in vivo* is considered to be essential for the optimization of drug therapy (Levy, 1983; Holford & Sheiner, 1982). Despite the advances in analytical methodologies to measure drug and metabolite concentrations, the lack of appropriate, objective measures of pharmacological effect intensity has limited the characterization of these relationships, especially for drugs which act on the central nervous system (CNS) (Dingemanse *et al.*, 1988a). Recently, quantitative EEG effect parameters have been shown to provide continuous, objective, reproducible and sensitive measures of the central nervous system effects of benzodiazepines, allowing the characterization of *in vivo* concentration-effect relationships of these drugs (Koopmans *et al.*, 1988; Greenblatt *et al.*, 1989; Breimer *et al.*, 1990; Mandema *et al.*, 1991b,c). However, the relevance of the EEG parameters with respect to the pharmacological effects of benzodiazepines in terms of potency and intrinsic efficacy remains to be established.

The mechanism of action of benzodiazepines is well known and involves their specific interaction with the γ-aminobutyric acid (GABA)-benzodiazepine receptor complex and their ability to enhance GABA-induced inhibitory action (Haefely *et al.*, 1985; Haefely, 1989). During the past few years, compounds have been discovered that cover the whole spectrum of intrinsic efficacy ranging from the traditional full agonists to inverse agonists (Braestrup *et al.*, 1983; Gardner, 1988; Haefely, 1988; 1989). This continuum includes partial (inverse) agonists, which produce only a part of (inverse) agonistic activity and antagonists, which selectively antagonize the effects of both (partial) agonists and inverse agonists without producing any intrinsic effects by themselves. Therefore, it is essential to evaluate for validity reasons whether EEG effect parameters reflect the interaction of benzodiazepines with and their intrinsic efficacy at the GABA-benzodiazepine receptor complex. In a previous study a close correlation between the potency of different benzodiazepine agonists with respect to their EEG effects, their receptor affinity and anticonvulsant effects as determined by the pentylenetetrazol (PTZ)-induced seizure threshold model was found (Mandema *et al.*, 1991b). The purpose of the present investigation was to quantify the concentration-EEG effect relationship of a benzodiazepine agonist midazolam, partial agonist bretazenil (Ro 16-6028), antagonist flumazenil and inverse agonist Ro 19-4603 (Figure 1) and to determine whether their differences in intrinsic efficacy are adequately reflected in pharmacodynamic parameters derived from EEG effect measures.

Methods

Animals

Male Wistar derived rats (225–275 g) were used throughout the study. The animals were housed individually in plastic cages with a normal 12 h light-dark cycle and fed on a commercially available diet (Standard Laboratory Rat, Mouse and Hamster Diets, RMH-TM, Hope Farms, Woerden, The Netherlands) and water *ad libitum*. From the night before experimentation the animals were deprived of food but had free access to water.

Pharmacokinetic-pharmacodynamic experiments

For the measurement of EEG signals, seven cortical electrodes were chronically implanted into the skull of the animals one week before the kinetic-dynamic experiments as previously

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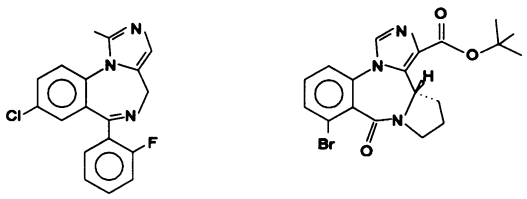

Figure 1 Chemical structures of the benzodiazepine agonist midazolam, partial agonist bretazenil (Ro 16-6028), antagonist flumazenil and inverse agonist Ro 19-4603.

described (Mandema & Danhof, 1990). Indwelling cannulae were implanted into the right jugular vein (for drug administration) and right femoral artery (for sample collection) under light ether anaesthesia, one day before the experiment. Dimethylacetamide (DMA) was used to make intravenous administration of the water insoluble benzodiazepines possible (Dingemanse *et al.*, 1988b). Drugs were dissolved at a concentration to give a dose volume of 100 μl of DMA. Rats were randomly assigned to four treatment groups that each received 2.5 mg kg^{-1} bretazenil, 10 mg kg^{-1} flumazenil, 2.5 mg kg^{-1} Ro 19-4603 or 100 μl DMA (placebo) intravenously, administered in 2 min by an infusion pump. The pharmacokinetic and pharmacodynamic data of midazolam were the same as found in a previous study (Mandema *et al.*, 1991b). Arterial blood samples (100–200 μl) were taken at intervals and immediately diluted with 0.5 ml of ice-cold 0.42% sodium fluoride solution in water and kept on ice to inhibit esterase activity. The diluted blood samples were stored at −35°C until analyzed for drug concentrations.

During the course of the experiment the animals had to walk in a slowly rotating drum (10 r.p.h.) to control the level of vigilance and prevent the animals from falling asleep spontaneously. EEG recordings were started 15 min before drug administration for baseline determination and continuously monitored by use of a system 50000 EEG recorder (Van Gogh BV, Amsterdam, The Netherlands). Following injection of the drug, the EEG recordings were continued until the signals had returned to pre-administration values. Two EEG leads, fronto-central and central-occipital on the left hemisphere, were subjected to on-line aperiodic analysis for quantification (Gregory & Pettus, 1986). The benzodiazepine induced change from baseline in the amplitudes (μVs^{-1}) in the 11.5–30 Hz (beta) frequency band of the fronto-central lead were calculated and used as a measure of drug effect (Mandema *et al.*, 1991b,c). The effect data points were calculated by averaging at least 60 s of consecutive EEG data. More frequent measurements were taken at the time of rapid change in drug concentration, e.g. immediately after administration of the bolus dose.

Distribution between blood and CNS

The distribution of flumazenil and midazolam between blood, CSF and brain was determined after administration of a cocktail of 10 mg kg^{-1} midazolam and 5 mg kg^{-1} flumazenil to a group of 7 animals weighing 200–250 g. CSF (by a puncture of a cisterna magna), blood (by an aortic puncture) and brain tissue were collected at 30 min after drug administration. Samples of 100 μl of blood were taken and treated as described earlier for midazolam and flumazenil blood concentration determination. The remainder of the blood was centrifuged and plasma was removed to determine midazolam plasma concentrations (100 μl sample). The brains were excised from the crania and one hemisphere was weighed and gently homogenized in 3 ml of ice-cold 0.42% sodium fluoride solution in water and rapidly stored at −35°C until analysis. Samples of 200 μl of brain homogenate were used to determine midazolam and flumazenil brain concentrations. The distribution between blood, CSF and brain of Ro 16-6028 and Ro 19-4603 was determined accordingly after administration of a cocktail of 5 mg kg^{-1} Ro 16-6028 and 5 mg kg^{-1} Ro 19-4603 to a group of 10 rats weighing 225–275 g. As protein binding of several benzodiazepines has been shown to be independent of concentration over a wide concentration range (0.01–10 mg l^{-1}) (Moschitto & Greenblatt, 1983), no interaction was expected with respect to the distribution to the CNS when the drugs were administered together.

Drug analysis

The blood, CSF and brain concentrations of flumazenil and midazolam were determined by a specific high pressure liquid chromatographic assay (h.p.l.c.) using u.v. detection, as previously described (Mandema *et al.*, 1991a,c). A sensitive assay was developed to measure the concentrations of bretazenil and Ro 19-4603 in small blood samples by h.p.l.c. and u.v. detection. A solution of 20 ng diazepam (internal standard) in 20 μl methanol was added with NaF solution to the diluted blood samples. Subsequently the samples were further diluted with 0.5 ml 0.1 N sodium hydroxide and extracted with 5 ml dichloromethane-pentane (1:1) for 30 s on a vortex mixer. After centrifugation the organic layer was separated and evaporated to dryness under reduced pressure. The residue was reconstituted with 200 μl mobile phase of which 100 μl was injected into the chromatographic system. The chromatographic system consisted of a M-45 solvent delivery pump, a WISP 710B automatic injector, a 10 cm μBondapack C18 column and a Lambda-Max model 418 LC spectrophotometer (all of Waters), which was set at 235 nm for bretazenil and 250 nm for Ro 19-4603. The mobile phase for bretazenil consisted of a mixture of 0.01 M phosphate buffer, pH = 5.0, and methanol in a ratio 35/65, with a flow rate of 1.0 ml min^{-1}. The mobile phase for Ro 19-4603 consisted of a mixture of 0.01 M phosphate buffer, pH = 5.0, and methanol in a ratio 40/60, with a flow rate of 0.8 ml min^{-1}. The within and between day coefficients of variation were less than 6% for Ro 19-4603 and less than 8% for bretazenil in the concentration range studied. The detection limit was about 5 ng ml^{-1} for both drugs, when a 200 μl blood sample was used. The brain and CSF samples were treated in similar manner to the blood samples.

Bretazenil and Ro 19-4603 were found to be stable in fresh rat blood *in vitro* kept at 37°C for 4 h.

Receptor binding

Binding affinities to the central GABA benzodiazepine receptor complex were determined based on displacement of [^{3}H]-flumazenil (New England Nuclear-757, specific activity 78.0 Ci mmol^{-1}) in a washed brain homogenate at 37°C as described previously (Mandema *et al.*, 1991b). Briefly, brains were obtained from drug-naive rats kept under similar conditions as described above. Total brains minus brain stems were gently homogenized in 10 vol of ice-cold, 'enriched' Tris buffer, containing 50 mM Tris, 120 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$ and 1 mM $MgCl_2$ (pH = 7.4). The homogenate was centrifuged at 48,000 *g* for 20 min and the supernatant was discarded. The pellet was resuspended in 10 vol of the 'enriched' Tris buffer. The procedure was repeated four times after which the homogenate was rapidly frozen and stored at −70°C. Brain homogenate aliquots (250 μl), to which GABA was added at a concentration of 33 μM and containing a protein concentration of 1.5 mg ml^{-1}, were incubated at 37°C with

approximately 6.4 ng ml^{-1} [^{3}H]-flumazenil and various concentrations of the displacing ligands. Alcohol, necessary to dissolve the benzodiazepines, was fixed at a concentration of 0.3% (Hollander-Jansen *et al.*, 1989). After equilibrium was reached (5 min) the samples were filtered through a presoaked glass-fibre filter (Whatman GF/B) under mild suction (Millipore 1225 Sampling Manifold). The filter was immediately washed twice with 5 ml of ice-cold, 'enriched' Tris buffer after which radioactivity was measured. Specific binding was assessed by subtracting the non-specific binding, obtained in the presence of 10 μg ml^{-1} flumazenil, from the total binding.

The receptor binding characteristics (K_d and B_{max}) of the radioligand [^{3}H]-flumazenil were determined in a saturation experiment under similar conditions as described above in the concentration range of 0 to 200 ng ml^{-1}. Free radioligand concentrations were calculated by subtracting the concentration of totally bound [^{3}H]-flumazenil at equilibrium from the concentration of [^{3}H]-flumazenil added to the incubation mixture. In both the displacement and saturation experiments binding was determined in triplicate at each concentration.

Data analysis

The pharmacokinetics of the different benzodiazepines were quantified for each individual rat. The plasma concentration time profiles were described by a poly-exponential equation:

$$C(t) = \sum_{i=1}^{n} A_i(e^{-\alpha_i t}) \quad (1)$$

where C(t) is the plasma concentration at time t and A_i and α_i are respectively the coefficients and exponents of the equation. Different exponential models were investigated and the most suitable model was chosen according to the Akaike information criterion (Akaike, 1974; Yamaoka *et al.*, 1978). Basic pharmacokinetic parameters such as clearance (Cl), volume of distribution at steady-state (Vdss) and mean residence time (MRT) were calculated from the coefficients and exponents of the fitted functions by standard methods (Gibaldi & Perrier, 1982).

The sigmoidal E_{max} model was used to describe the relationship between drug concentration and EEG effect (Holford & Sheiner, 1982):

$$E(C) = \frac{E_{max} \cdot C^N}{EC_{50}{}^N + C^N} \quad (2)$$

where E(C) is the observed effect (change from baseline) at concentration C, E_{max} is the maximal effect, EC_{50} is the concentration at half maximal effect and N is a constant expressing the shape of the concentration-effect relationship. The pharmacokinetic model was used to generate the concentrations of the drug at the times of effect measurement.

The receptor binding characteristics of [^{3}H]-flumazenil as determined in the saturation experiment have already been presented in a previous study (Mandema *et al.*, 1991b). IC_{50} values of the benzodiazepines were determined by fitting the following equation to the displacement data:

$$B = \frac{B_o \cdot IC_{50}}{IC_{50} + C_d} \quad (3)$$

In which B_o is the specific binding of the radioligand with no displacer present and C_d is the concentration of displacer added. K_i values were derived from the IC_{50} values according to the Cheng-Prusoff equation:

$$K_i = \frac{IC_{50}}{1 + \frac{L}{K_d}} \quad (4)$$

in which L is the concentration of radioligand used in the displacement studies. The equations were fitted to the data by use of the non-linear least squares regression program Siphar (Simed SA, Creteil, France). Statistical analysis was conducted by a one-way analysis of variance. A significance level of 5% was selected.

Results

Pharmacokinetics

The time course of drug concentrations after intravenous administration of the four benzodiazepines is shown in Figure 2. The solid lines represent the best fits of the pharmacokinetic model to the pooled data of all animals within one treatment group. For all four drugs, a bi-exponential equation (equation 1) was found to best characterize the data.

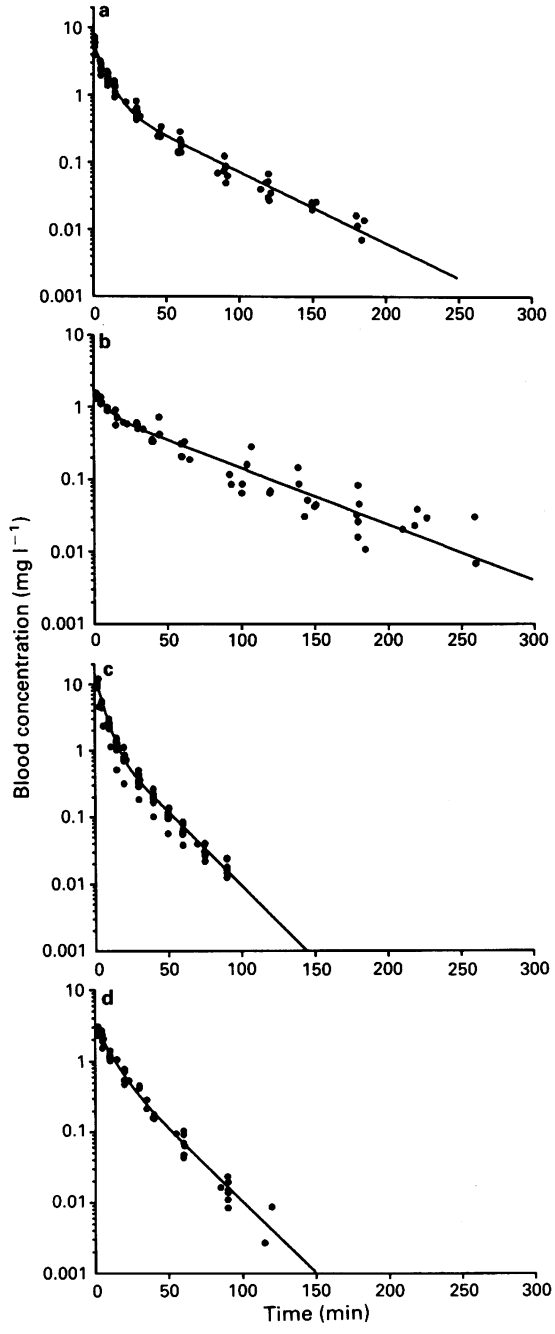

Figure 2 Blood concentration (●) versus time profiles of all individual animals, which had received an intravenous bolus administration of (a) 5 mg kg^{-1} midazolam, (b) 2.5 mg kg^{-1} bretazenil, (c) 10 mg kg^{-1} flumazenil or (d) 2.5 mg kg^{-1} Ro 19-4603. The solid lines represent the best fits of the pharmacokinetic model to the data.

Table 1 Pharmacokinetic parameter estimates of the four benzodiazepines

	Midazolam	*Bretazenil*	*Flumazenil*	*Ro 19-4603*
Number of animals	7	7	8	7
Cl (ml $min^{-1} kg^{-1}$)	92 ± 4	47 ± 3	114 ± 13	65 ± 6
Vdss (l kg^{-1})	2.2 ± 0.1	2.2 ± 0.1	1.1 ± 0.1	0.98 ± 0.09
MRT (min)	24 ± 1	48 ± 4	9.7 ± 0.2	15.0 ± 0.8

Cl, clearance; Vdss, volume of distribution at steady-state; MRT, mean residence time. The pharmacokinetic parameters are based on blood concentrations. The values are presented as means ± s.e.mean. Significant differences between the four drugs were found for Cl, Vdss and MRT (ANOVA, $P < 0.05$).

The pharmacokinetic parameters of each drug were calculated for the individual animals and are summarized in Table 1. Midazolam concentrations were originally measured in plasma (Mandema *et al.*, 1991b). The plasma to blood concentration ratio of midazolam was 1.23 ± 0.05 ($n = 7$), which was used to calculate the pharmacokinetic parameters of midazolam based on blood concentrations shown in Table 1. The pharmacokinetic parameters showed significant differences between the four drugs. The clearance value ranged from 114 ml $min^{-1} kg^{-1}$ for flumazenil to 47 ml $min^{-1} kg^{-1}$ for bretazenil and the mean residence time ranged from 9.7 min for flumazenil to 48 min for bretazenil.

The parameters describing the distribution of the four benzodiazepines between blood and the CNS in steady-state are shown in Table 2. Large differences were found between the CSF to blood concentration-ratio for the four drugs, with midazolam exhibiting the lowest value of 0.050 and flumazenil the highest value of 0.526. The differences in blood-brain distribution were much less pronounced.

Pharmacodynamics

The administration of the solvent DMA produced a small reduction in the EEG effect measure (amplitudes in the 11.5–30 Hz frequency band). However, it was large enough to distort the relatively small effects of the partial agonist, antagonist and inverse agonist. Therefore, the EEG effect versus time profiles of all individual animals were corrected for the averaged vehicle effect response. The corrected changes in EEG effect measure as a function of time for the four benzodiazepines are shown in Figure 3. This figure shows the averaged changes in EEG effect of all rats within one treatment group. For clarity the s.e.mean values are omitted. Figure 3 clearly shows the large differences in intrinsic efficacy of the four compounds. Midazolam produced the largest increase in EEG effect, bretazenil produced a much smaller increase, whereas Ro 19-4603 produced a small but significant decrease in the amplitudes in the 11.5–30 Hz frequency band. Flumazenil produced an initial increase in the EEG effect measure, which only lasted for 3 min, after which time no significant differences from the baseline EEG values were observed for the remainder of the registration period. No hysteresis was observed between plasma/blood concentrations and EEG effects for all four drugs and the values were directly correlated to each other. Figure 4 shows the averaged concentration-EEG effect relationship of the four drugs. The pharmacokinetic fits shown in Figure 2 were used to calculate the drug concentrations at the time points of effect measurement. The concentration-EEG effect relationships of midazolam, bretazenil and Ro 19-4603 were successfully quantified by the sigmoidal E_{max} model, as is shown by the solid lines in Figure 4. Flumazenil only produced a statistically significant increase in the EEG effect parameter at concentrations above 8 mg ml^{-1}. The peak effect after administration of flumazenil was 26 ± 3 μVs^{-1} at a concentration of 13 mg l^{-1}. In the concentration range of 0.1 mg l^{-1} to 1 mg l^{-1} the averaged effect was 0.7 ± 0.3 μVs^{-1} and not significantly different from baseline.

The concentration-EEG effect relationship of midazolam and bretazenil could be adequately quantified in individual animals and the estimated pharmacodynamic parameters are summarized in Table 3. The concentration-EEG effect relationship of the inverse agonist could not be adequately quantified by the sigmoidal E_{max} model in the individual animals. The pharmacodynamic parameters of Ro 19-4603 shown in Table 3 were estimated by fitting the sigmoidal E_{max} model to the averaged concentration-effect data and represent the parameter estimates of the fit and the estimates of the standard error of the parameter estimates. Since the pharmacodynamic parameters of the drugs were obtained in a different manner a statistical comparison with ANOVA was not possible and multiple t tests were used assuming the estimate of s.e.mean to be a population value. The maximal effects of midazolam, bretazenil and Ro 19-4603 showed significant differences. The EC_{50} values, based on total blood concentrations and CSF concentrations of bretazenil and Ro 19-4603,

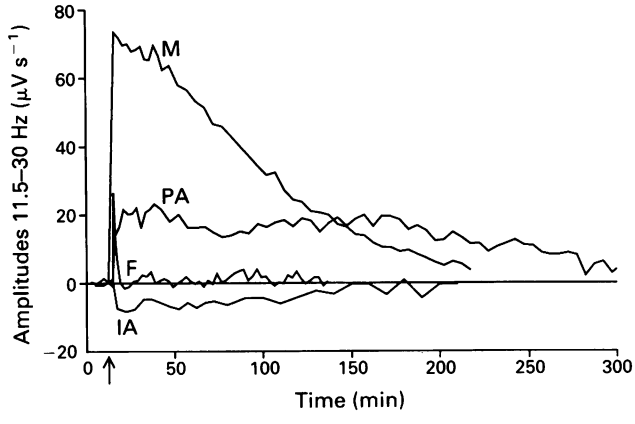

Figure 3 Averaged EEG effect (change in amplitudes in the 11.5–30 Hz frequency band) versus time profiles of all individual animals, which had received an intravenous bolus administration, marked by the arrow, of 5 mg kg^{-1} midazolam (M), 2.5 mg kg^{-1} bretazenil (PA), 10 mg kg^{-1} flumazenil (F) or 2.5 mg kg^{-1} Ro 19-4603 (IA).

Table 2 Distribution parameters of the four benzodiazepines between blood and the central nervous system

	Midazolam	*Bretazenil*	*Flumazenil*	*Ro 19-4603*
Number of animals	7	10	7	10
CSF/blood	0.050 ± 0.005	0.115 ± 0.006	0.526 ± 0.045	0.279 ± 0.008
Brain/blood (ml g^{-1})	2.10 ± 0.06	0.82 ± 0.02	0.51 ± 0.02	0.56 ± 0.01

The values are presented as means ± s.e.mean. Significant differences between the four drugs were found for the CSF to blood and brain to blood concentration-ratios (ANOVA, $P < 0.05$).

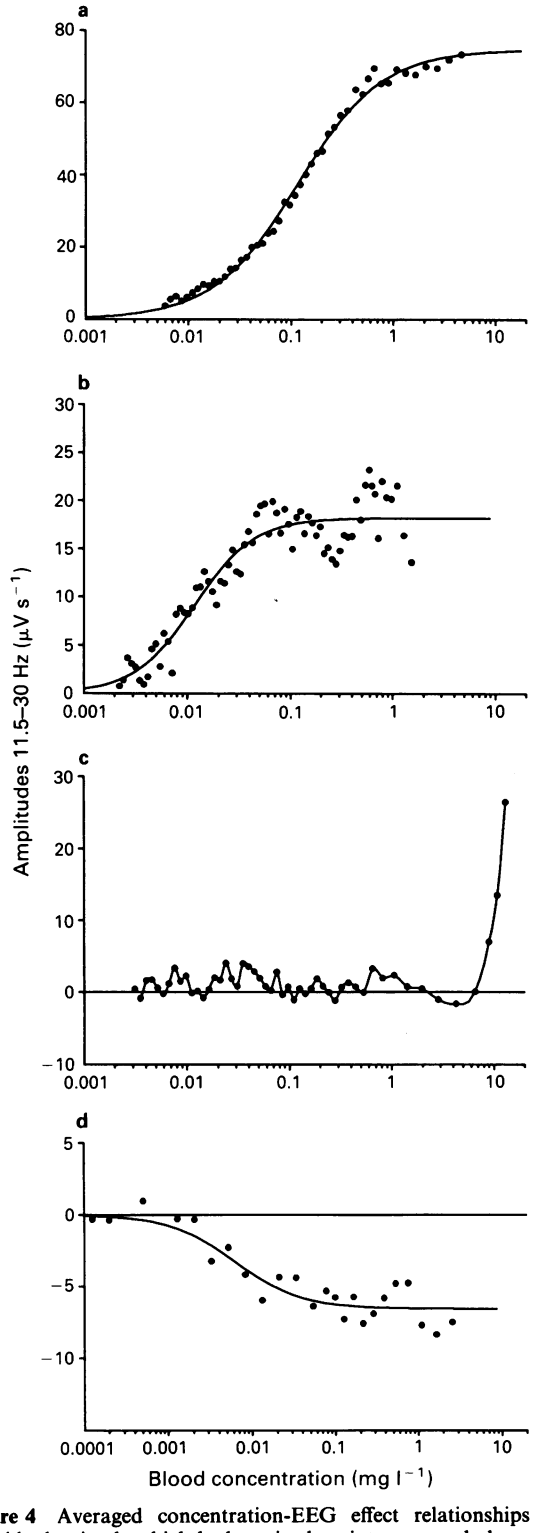

Figure 4 Averaged concentration-EEG effect relationships of all individual animals which had received an intravenous bolus administration of (a) 5 mg kg^{-1} midazolam, (b) 2.5 mg kg^{-1} bretazenil, (c) 10 mg kg^{-1} flumazenil or (d) 2.5 mg kg^{-1} Ro 19-4603. The sigmoidal E_{max} model was fitted to the concentration-effect data of midazolam, bretazenil and Ro 19-4603. Note the different scales on the y-axis.

were not statistically significantly different from each other. However, the values were significantly lower than the corresponding value for midazolam.

The receptor binding parameters of [^{3}H]-flumazenil as determined in the saturation experiment at 37°C were K_d = 7.1 ± 1.5 ng ml^{-1} and B_{max} = 0.545 ± 0.025 ng mg^{-1} protein (Mandema *et al.*, 1991b). For all benzodiazepines evaluated in the displacement studies the addition of a slope factor N in equation 3 did not improve the 'goodness of fit', indicating the presence of only one binding site. This assumption is the basis of the Cheng-Prusoff equation used to derive the K_i values from the IC_{50} values. The validity of the used models is further exemplified by the fact that the K_i value (6.8 ± 1.3 ng ml^{-1}) of flumazenil determined in the displacement studies was identical to the K_d value calculated from the saturation data. In Table 3 the receptor affinity, presented by their K_i values, is given for the three compounds exhibiting an intrinsic effect. For all three benzodiazepines the CSF concentration required to produce 50% of the maximal EEG effect is in the same range as the K_i values.

Table 3 Pharmacodynamic parameter estimates of the benzodiazepines

	Midazolam	*Bretazenil*	*Ro 19-4603*
Number of animals	7	7	7
E_{max} (μVs^{-1})	73 ± 2	19 ± 1	−6.5 ± 0.4
N	1.5 ± 0.2	2.0 ± 0.3	1.1 ± 0.4
EC_{50} (ng ml^{-1})	85 ± 8	12 ± 3	6.2 ± 1.9
$EC_{50,CSF}$ (ng ml^{-1})	4.3 ± 0.8	1.4 ± 0.4	1.7 ± 0.5
K_i[a] (ng ml^{-1})	4.9 ± 0.5	1.2 ± 0.2	1.5 ± 0.5

E_{max}, maximal effect; N, sigmoidicity of concentration-effect relationship; EC_{50}, blood concentration producing half-maximal effect; $EC_{50,CSF}$, CSF concentration producing half-maximal effect; K_i, concentration producing 50% receptor occupancy. The values are presented as means ± s.e.mean.
[a] The K_i values were determined based on displacement of [^{3}H]-flumazenil *in vitro* at 37°C.

Discussion

The characterization of relevant relationships between the pharmacokinetics and pharmacodynamics of benzodiazepines requires the availability of appropriate measures of drug effects. Recently, quantitative EEG measures were shown to characterize successfully the concentration-EEG effect relationships of various benzodiazepine agonists (Koopmans *et al.*, 1988; Greenblatt *et al.*, 1989; Breimer *et al.*, 1990; Mandema *et al.*, 1991b,c). The EEG effect measures appear to fulfil many of the characteristics of ideal pharmacodynamic measures, being continuous, objective, sensitive and reproducible (Dingemanse *et al.*, 1988a). However, the pharmacological relevance of EEG parameters with respect to the various CNS effects of benzodiazepines remains to be established. The anxiolytic, anticonvulsant, muscle relaxant and sedative-hypnotic actions of benzodiazepines result from the interaction of these compounds with the GABA-benzodiazepine receptor complex and their ability to modulate GABA-induced inhibitory actions (Möhler & Okada, 1978; Haefely *et al.*, 1985; Haefely, 1989). The modulation of GABAergic activity by benzodiazepines is quite unique because of the fact that compounds have been discovered that cover the whole spectrum of intrinsic efficacy ranging from full agonists, enhancing GABAergic activity, to inverse agonists, which reduce the effects of GABA. This continuum also includes partial agonists, antagonists and partial inverse agonists (Braestrup *et al.*, 1983; Gardner, 1988; Haefely, 1988; 1989). In the present investigation the concentration-EEG effect relationships of four benzodiazepine ligands with large differences in their intrinsic efficacy at the GABA-benzodiazepine receptor complex were characterized.

The benzodiazepines studied were selected on the basis of their known differences in intrinsic efficacy. Midazolam is an extensively studied benzodiazepine ligand, which exhibits the pharmacological profile of a full agonist (Pieri *et al.*, 1981). Flumazenil was shown to antagonize selectively the central effects of benzodiazepines without possessing any intrinsic effects of its own (Hunkeler *et al.*, 1981; Polc *et al.*, 1981; Bonetti *et al.*, 1982). The partial agonistic activity of bretazenil was shown in a variety of tests (Haefely, 1984; Martin *et al.*,

1988; Potier *et al.*, 1988; Haefely, 1988; Yakushiji *et al.*, 1989). Interestingly, this drug was found to possess potent anxiolytic and anticonvulsant properties, but much less sedative and motor impairment activity (Martin *et al.*, 1988; Haefely, 1988; Haefely *et al.*, 1990). Ro 19-4603 was found to be a potent inverse agonist exhibiting a specific high affinity binding to the central benzodiazepine receptor comparable to that of bretazenil and was shown to possess intrinsic anxiogenic and proconvulsant properties (Pieri, 1988; Belzung *et al.*, 1990).

The benzodiazepine-induced changes in amplitudes in the 11.5–30 Hz (beta) frequency band were used as EEG effect measure. In a previous study a close correlation was found between the free drug concentrations of four benzodiazepine agonists required to produce 50% of their maximal EEG effect and the concentrations required to produce 50% receptor occupancy, as determind in an *in vitro* displacement study at 37°C (Mandema *et al.*, 1991b). In the present study it was clearly shown that differences in intrinsic efficacy of the benzodiazepines are also adequately reflected in the EEG effect parameter (Figures 3 and 4; Table 3). The concentration-EEG effect relationships of midazolam, bretazenil and Ro 19-4603 were successfully quantified by the sigmoidal E_{max} model and to our knowledge this is the first study that established these relationships for a partial agonist and inverse agonist *in vivo*. The concentration-EEG effect relationship of the inverse agonist could not be adequately quantified by the sigmoidal E_{max} model in the individual animals. This is probably due to the relatively small effects of this drug with respect to the normal fluctuations in the EEG effect measure independent of drug concentrations. The pharmacodynamic parameters of midazolam and bretazenil derived from the averaged concentration-EEG effect relationships did not differ from the parameters presented in Table 3. The averaged maximal effect observed for the partial agonist bretazenil was $19\,\mu V\,s^{-1}$ and substantially less than the maximal effect of midazolam (Table 3). Studies on the relative intrinsic efficacy of bretazenil with respect to full agonists are relatively scarce. However, the maximal increase of the convulsant threshold dose of pentylenetetrazol by bretazenil is about one quarter of that achieved with the full agonist diazepam (Haefely, 1990), which is similar to the ratio found in this study between the maximal effects of bretazenil and midazolam. In a previous study a close agreement between the pharmacodynamic parameters (EC_{50} and relative E_{max}) obtained in the EEG model and pentylenetetrazol convulsant threshold model was also found (Mandema *et al.*, 1991b). The inverse agonist Ro 19-4603 produced a decrease in the EEG effect measure with a maximal effect of $-6.5\,\mu V\,s^{-1}$. This decrease is relatively small, but significantly different from baseline. That only a small decrease was produced by Ro 19-4603 may be caused by the fact that the EEG effects were measured under controlled vigilance conditions. Under these conditions, the additional vigilance enhancing effects of the inverse agonist may only be small. This is in accordance with the observations made by Ongini *et al.* (1983), who showed that the inverse agonist FG 7142 increased the state of alertness and elicited EEG changes (decrease in beta power) in cats especially when given during periods of drowsiness.

The EC_{50} values should be corrected for differences in protein binding in order to be able to compare the relative potencies of these drugs (Mandema *et al.*, 1991b). Plasma protein binding of flumazenil cannot be determined due to the instability of this compound in plasma at 37°C (Mandema *et al.*, 1991a). As an alternative, CSF concentrations were used as representative for the free drug concentration. Since benzodiazepines were shown to equilibrate rapidly between blood and CSF (Arendt *et al.*, 1983), the ratios determined 30 min after the intravenous administration may be considered similar to the steady-state values. The CSF to plasma ratio of midazolam was 0.041 ± 0.007 and similar to the fraction unbound in plasma of 0.037 ± 0.005 as determined in a previous study (Mandema *et al.*, 1991b), indicating that the CSF concentrations are more or less equal to the free drug concentrations in plasma. Large differences were found between the CSF to blood ratio of the four drugs. The EC_{50} values of midazolam, bretazenil and Ro 19-4603 based on CSF concentrations are in the same order of magnitude as the concentrations required to produce 50% receptor occupancy (K_i) and the same rank order of potency was found based upon the two measures (Table 3). No close correlation seemed to exist when total brain concentrations were used to correct the EC_{50} values.

Flumazenil produced a significant increase in the EEG effect measure at relatively high concentrations ($>8\,mg\,l^{-1}$). Since the original reports that flumazenil had no intrinsic efficacy (Hunkeler *et al.*, 1981; Polc *et al.*, 1981; Bonetti *et al.*, 1982), various studies have appeared in the literature claiming that flumazenil might possess some partial agonist and even inverse agonist properties (for reviews see File & Pellow, 1986; Brodgen & Goa, 1988), of which the partial agonist properties were mostly observed after administration of high doses. However, considering the high affinity of flumazenil for the central benzodiazepine receptor ($K_i = 6.8 \pm 1.3\,ng\,ml^{-1}$) and high CSF to plasma ratio, it is unlikely that the EEG effects produced by this drug are the result of the interaction of this drug with this receptor. In view of the results obtained for the other three compounds, any intrinsic effects of flumazenil due to interaction with the high affinity benzodiazepine receptor must occur in the concentration range when receptor saturation takes place. However, in the blood concentration range of 100 to $1000\,ng\,ml^{-1}$ (approximately 90 to 99% receptor occupation) the average effect of flumazenil was $0.7 \pm 0.3\,\mu V\,s^{-1}$ and not significantly different from zero. The mechanism of the increase produced by flumazenil is thus unclear. However, benzodiazepines have been shown to interact with several other neurotransmitter systems, especially at high doses (Morgan *et al.*, 1983; Haefely *et al.*, 1985).

Large differences between the pharmacokinetic parameters were observed (Table 1). Flumazenil was rapidly eliminated from the rat body with a mean residence time of only 9.7 min due to a large clearance value and a small volume of distribution. The mean residence time of Ro 19-4603 was somewhat longer due to the lower clearance value of this drug. The disappearance of bretazenil from the rat body was considerably slower with a mean residence time of 48 min, which can be explained by the lower clearance value and larger volume of distribution found for this drug when compared to the other four compounds (Table 1). Flumazenil was found to be highly instable in fresh rat blood kept *in vitro*, probably due to aspecific esterases present in rat blood (Mandema *et al.*, 1991a). However, bretazenil and Ro 19-4603 were found to be stable when kept in fresh rat blood *in vitro* at 37°C for 4 h, which is most likely due to the change of the ethyl-ester group of flumazenil to a t-butyl-ester group. This lower sensitivity to esterases may also explain the lower clearance values found for these drugs, although little is known about the metabolic pathway of these three compounds in rats.

In conclusion, the concentration-EEG effect relationships of the benzodiazepine agonist midazolam, partial agonist bretazenil, antagonist flumazenil and inverse agonist Ro 19-4603 were quantified. The differences in intrinsic efficacy of these compounds were reflected in the EEG effect parameter (change in amplitudes in the 11.5–30 Hz (beta) frequency band). Furthermore, a close correlation was found between the free drug EC_{50} values and receptor affinity. These results suggest that the change in amplitudes in the 11.5–30 (beta) Hz frequency band is a realistic measure of the CNS effects of benzodiazepines, reflecting their interaction with and intrinsic efficacy at the central GABA-benzodiazepine receptor complex.

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